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Selenized yeast has also recently been shown to reduce lung cancer incidence and mortality in a population of skin cancer patients. Smokers and survivors of early stage lung and head and neck cancers have had a long period of promotion by carcinogenic agents on the bronchial epithelium resulting in morphologic and molecular alterations. We hypothesize that these morphologic and molecular alterations can be detected and modulated by chemopreventive agents. We have proposed a Phase IIa chemoprevention trial evaluating five different dose levels of selenium administered daily for 3 months in subjects at high risk for lung cancer with bronchoscopically documented dysplasia. Subjects will be entered to determine the effect of dose on the modulation of biomarkers in response to selenium supplementation as well as to measure selenium levels and modulation of glutathione peroxidase as a measure of drug effect. In addition to morphology, the surrogate endpoint biomarkers to be examined include apoptosis, p53 expression, K-ras mutation analysis, p16 methylation, and upregulation of hnRNP A2/B1. Successful completion of this study will support selenium supplementation as potentially beneficial therapy in preventing the progression of lung carcinogenesis as well as identify surrogate endpoint markers that appear to be modulated by selenium supplementation.

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PHASE IIA CHEMOPREVENTION STUDY OF SELENIUM IN PERSONS AT RISK FOR LUNG CANCER

INTRODUCTION

Much knowledge has been acquired about the multi-step process of carcinogenesis in the lung during the last 20 years. Tumorigenesis appears to be the result of a number of genetic insults, although it remains to be determined whether there is a necessary sequence or a critical number of events required. Certain genetic alterations can be detected in the bronchial epithelium of persons at increased risk for lung cancer. Selenium may act through several different mechanisms of action, including stimulation of apoptosis, protection of tissue against oxidative damage, inhibition of tumor growth, reduction of mutagenic activity and reduction of activation of carcinogens and stimulation of the immune system. Selenized yeast has also recently been shown to reduce lung cancer incidence and mortality in a population of skin cancer patients. Smokers and survivors of early stage lung and head and neck cancers have had a long period of promotion by carcinogenic agents on the bronchial epithelium resulting in morphologic and molecular alterations. **We hypothesize that these morphologic and molecular alterations can be detected and modulated by chemopreventive agents.** We have proposed a Phase IIa chemoprevention trial evaluating five different dose levels of selenium administered daily for 3 months in subjects at high risk for lung cancer with bronchoscopically documented dysplasia. After establishing the maximum tolerated dose, additional subjects will be entered at that dose level in order to examine the modulation of biomarkers in response to selenium supplementation as well as to measure selenium levels and modulation of glutathione peroxidase as a measure of drug effect. In addition to morphology, the surrogate endpoint biomarkers to be examined include apoptosis, p53 expression, K-ras mutation analysis, p16 methylation, and upregulation of hnRNP A2/B1. Successful completion of this study will support selenium supplementation as potentially beneficial therapy in preventing the progression of lung carcinogenesis as well as identify surrogate endpoint markers that appear to be modulated by selenium supplementation.

BODY

The Statement of Work was submitted as a timeline. No secondary patient accrual was begun, and all patient accrual for completion of the initial accrual was stopped.

A total of 499 subjects have been screened for the study, with 153 eligible for the study. Of those eligible, 151 chose to participate in the study and sputum induction was performed. Forty-one were normal and 102 showed some abnormality, with 8 specimens showing inadequate levels of cells or no specimen could be produced. Fifty-three of those 102 subjects elected to continue on to the bronchoscopy. Analysis of bronchial biopsies resulted in 14 subjects being eligible to take part in the selenium dose selection phase of the study and all agreed to participate. All fourteen subjects have completed three months of selenium at the five dose levels.

Demographics

Of the 151 subjects who had a sputum induction, 96 were male and 55 female. The group consisted of 90 current smokers and 61 former smokers. Of the 53 subjects who proceeded to bronchoscopy, 41 were male and 12 were female. This group consisted of 23 current and 30 former smokers. Mean pack-years of smoking were 61.2 for males, 58.1 for females, 51.8 for former smokers and 72.4 for current smokers.

Sputum results

Sputum inductions on 151 subjects produced the following results:

Sputum Cytology Results	Number
No Significant Abnormality	41
Metaplasia	82
Mild Dysplasia	17
Moderate Dysplasia	2
Severe Dysplasia	1
Insufficient material/Not interpretable	8

The 102 subjects with metaplasia or dysplasia were eligible to proceed to bronchoscopy.

Bronchoscopy Results

Bronchoscopy was performed on 53 individuals. Those with dysplasia qualified to enter for selenium dose supplementation. Fourteen individuals were randomly assigned to one of five dose levels of selenium for 3 months and had a second bronchoscopy upon completion. The bronchoscopy results were as follows:

Bronchial Histopathology	Initial	3 Month
No Significant Abnormality	14	0
Basal Cell Hyperplasia	0	1
Regular Metaplasia	23	1
Mild dysplasia	11	8
Moderate Dysplasia	4	3
Severe Dysplasia	1	1
CIS	0	0
Invasive Cancer	0	0
Total	53	14

Data is presented by smoking status, atypia, and bronchoscopy findings in Figures 1-3.

Selenium Blood Levels

Blood samples taken from 14 selenium participants at baseline and at 3 months show increases in blood selenium levels for all subjects. Average selenium level at baseline was 130 mcg/l and at 3 months was 305.5 mcg/l. The selenium levels for those who did not proceed with the study were 131.7 mcg/l. None of the baseline levels of selenium suggest a dietary deficiency. An increase in the selenium methionine dose clearly shows an increase in the selenium levels measured in the blood. The 400 mcg/day dose can only be evaluated based on a single subject because one subject was dropped and an end of study blood sample was not collected for one subject. The end of study selenium levels at the 600 mcg/day dose suggests a higher level of variation between individuals than found in other doses. Both the 800 and 1000 mcg/day dose suggests less variation. This might be due to an individual not taking all of the drug throughout the study or could reflect an inherent variation among the individuals within the 600 mcg/day dose. One odd observation is that the end of study selenium levels within the 1000 mcg/day dose are lower than those found within the 800 mcg/day dose group. This observation does reach significance (Student's T test equals 3.51 with a probability of 0.0247). The reason for the drop in selenium levels at the highest dose is unknown, but might represent a physiological response to rid the body of the excess selenium. This could represent an induction of a molecular pathway to excrete the excess selenium that may not be effectively activated at the 800 mcg/day dose. Since the 800 mcg/day dose produces the highest levels of selenium in the blood, this dose would represent the maximum useful dose.

Selenium Dose in mcg/day	Patient	Baseline Selenium in mcg/liter	End of Study Selenium in mcg/liter	Change in Selenium mcg/liter	Average Change in Selenium
200	14	150	250	100	83
	31	160	220	60	
	130	100	190	90	
400	48	110	320	210	210
	140	140	Not Done		
	52	Dropped Out	NA		
600	82	140	200	60	130
	20	100	230	130	
	146	110	310	200	
800	39	180	425	245	285
	78	100	460	360	
	145	170	420	250	
1000	10	120	385	265	245
	4	140	380	240	
	136	110	340	230	

Adverse Events

A total of eight adverse event reports were filed, none of which were determined to be study related. Two adverse events reported for study subject number 146 were thought to be an

outcome of either anesthesia or dehydration from fasting for the bronchoscopy. A summary of the events is shown below.

Patient ID	Date of Event	Event	Study Related	Outcome
003	5/01/98	Asthma	No	Resolved
010	8/18/98	Cough, chest tightness, fever	No	Resolved
010	8/25/98	Follow-up	No	Resolved
074	12/17/98	Vaso-vagal response, post bronchoscopy	Possibly	Resolved
014	1/10/99	Hernia	No	Herniorrhaphy
014	2/01/99	Fever	No	Resolved
146	12/3/99 to 12/5/99	Migraine headache	Possibly	Resolved
146	8/6/99 to 8/9/99	Migraine headache	Possibly	Resolved

No selenium dependent toxicity was reported for any dose level.

Laboratory Progress Report

For the 14 individuals who have completed the study:

- 140 biopsies have been formalin fixed and paraffin embedded, with all blocks sectioned and stored at the Cancer Prevention Laboratory.
- 72 biopsy samples have been micro-dissected for collection of atypical and normal cells from the same site.
- 28 blood samples, with GPX assays done in triplicate including control assays for no substrate, no sample and a positive control with purified GPX (Figure 7) as well as hemoglobin levels (Figure 6).
- Methylation specific PCR (MSP) of the p16 gene has been done on both the baseline and post-selenium sputum samples. No aberrant methylation was detected.
- MSP has been done on DNA extracted from bronchial cells collected at the biopsy site but failed to attach to the culture dishes. To date, 192 samples have been tested for MSP of the p16 gene. Using control primers, 158 of the samples could be analyzed (82.3%) but only one sample showed a positive result for MSP of the p16 gene (0.52%). This sample, from an end of study bronchoscopy (031), showed hypermethylation of the p16 gene. No hypermethylation was detected in any of the other samples.
- All bronchial biopsy specimens for both the baseline and 3 month samples were immunostained for altered p16 expression. No obvious change in the staining pattern was seen between the two samples at the same biopsy site for the same individual. The subject with a positive hypermethylation result showed no obvious change or reduction in the p16 protein staining intensity at the same biopsy site.
- Assays for the detection of K-ras mutation in codon 12 proved unreliable and were discontinued.
- An assay was developed for the detection of over-expression of the p53 protein using standard immunohistochemistry on sputum samples. Sputum samples for the fourteen

individuals who have completed the study were tested. No increased p53 expression could be found.

- A standard TUNEL assay was used to detect apoptosis in the bronchial biopsies. No apoptotic cells were detected in any of the samples although all controls performed well.
- Proliferation assay based on immunostaining for Ki-67 were tested and discarded because of unreliable positive control. Proliferation assay based on immunohistochemical staining for the Proliferation Cell Nuclear Antigen (PCNA) was substituted. No difference in staining intensity or location of stain for PCNA was detected between the baseline and 3-month biopsy specimens from the same individual
- A subset of the biopsy samples was evaluated with an antibody directed against GADD 153, one of the proteins involved growth arrest in cells. No obvious difference was detected between biopsies collected before and after selenium supplementation.
- A subset of the biopsy samples were evaluated with an antibody directed against p27, a cyclin dependent kinase inhibitor. No obvious difference was detected between biopsies collected before and after selenium supplementation.
- A subset of the biopsy samples were evaluated with an antibody directed against FasL, part of the initial signaling for an apoptosis cascade. No obvious difference was detected between biopsies collected before and after selenium supplementation.
- A subset of the biopsy samples was evaluated with two different antibodies directed against thioredoxin reductase, a selenium containing enzyme. No obvious difference was detected between biopsies collected before and after selenium supplementation.

For all study subjects

- Tissue culture of normal bronchial epithelial cells on 53 study subjects
- 283 NHBE cultures have been attempted with 139 growing well enough for the collection of cells for the preparation of DNA or stored for regrowth.
- DNA has been prepared from either the cells grown in culture or from cells that failed to attach to the culture dish.
- 53 blood samples processed for isolation of lymphocytes and for the baseline measurement of GPX plus 14 additional samples from individuals who completed selenium supplementation (Figure 7).
- All samples, whole blood and lymphocytes, are stored at -80°C. All NHBE cultures are stored in liquid nitrogen.
- All biopsy blocks were initially stored and controlled by the Pathology Department of the H. Lee Moffitt Cancer Center. In general, for most study subjects the number of biopsies is the same as the number of sites used for collecting NHBE for cell culture. For a few study subjects, extra biopsies were taken at the discretion of the physician.
- Comparison of the interpretation of the pathology findings by our study pathologist (Dr. Khoor) with those of a blinded consultant (Dr. Gazdar) has been completed. No significant difference was found (Figure 4).

KEY RESEARCH ACCOMPLISHMENTS

- Established feasibility of recruiting and enrolling heavy current and former smokers on a chemoprevention study

- Developed algorithm to recruit and screen subjects, obtain induced sputum specimens, obtain history and physical and screening chest x-ray and blood work prior to bronchoscopy, obtain bronchoscopy and start eligible subjects on selenium supplement.
- Developed close collaborative relationships with pulmonary medicine and pathology
- Evaluated induced sputa from high risk individuals for p16 hypermethylation (all with no hypermethylation detected)
- Developed archive of 274 bronchial epithelial cell cultures
- Developed archive of induced sputum specimens from 151 high risk individuals
- Evaluated the value of fluorescent bronchoscopy in addition to white light bronchoscopy in predicting dysplasia in a high risk population (Figure 5).
- Measured glutathione peroxidase pre- and post-selenium supplementation and found no change
- Measured selenium pre- and post-selenium supplementation and found the blood selenium levels increased with increasing dose
- The blood selenium levels at the 800 mcg/day dose were higher than those in the 1000 mcg/day dose. This observation was found to be significant. (Student's T test equals 3.51 with a probability of 0.0247).

REPORTABLE OUTCOMES

- Development of repository of induced sputum specimens from 151 individuals
- Development of repository of bronchial epithelial cell cultures (274 cultures from 53 study subjects).
- Serum and lymphocyte repository from the same patient population.
- Fluorescent light bronchoscopy better predicts atypia than white light bronchoscopy (Figure 5).
- No p16 hypermethylation was detected in DNA samples from sputum collected at baseline for the 14 individuals who received selenium supplementation nor could we detect changes in the protein levels in biopsy specimens by immunostaining for p16.
- NHBE cells cultured in the laboratory were tested for the presence of hypermethylation of the p16 promoter region. From a total of 192 cultures tested for hypermethylation of the p16 promoter, we found that 158 cultures could be amplified (82.3%) with only one culture positive (0.52%).

CONCLUSIONS

The dose levels of selenium used in the initial phase of the protocol appear to have no obvious toxicity. The blood selenium levels increased after supplementation with selenomethionine. Baseline levels range from 92-200 μg/l and the levels after supplementation range from 190-460 μg/l. The increased levels of selenium correspond to the increasing doses of selenomethionine. The maximum level of blood selenium can be achieved by a dose of 800 mcg/day.

Based on our current analysis, fluorescent bronchoscopy is better able to detect atypia than white light bronchoscopy. This analysis confirms the initial anecdotal comments we were receiving from the bronchoscopy physicians and personnel.

Analysis of the hypermethylation of the p16 promoter region has, so far, not shown any significant alteration of the region to suggest repression of transcription. We failed to detect a methylation specific PCR product in the sputum samples but did detect a hypermethylated p16 allele in one sample of cells cultured from a bronchial brushing from a single site at the end of study bronchoscopy. We cannot conclude that the single hypermethylation event was caused by the selenomethine supplementation. Since hypermethylation of the p16 gene would result in turning off the tumor suppressor gene, this does not seem to fit with the proposed role of selenomethionine as a chemopreventive agent. The suppression of the p16 gene may have been a result of the cells adapting to cell culture. Even though the assay was repeated, we cannot eliminate the possibility of contamination of a single sample during that series of experiments but it seems unlikely since none of the other samples showed hypermethylation. We have completed the immunostaining of biopsy specimens for the expression of p16 protein and have not seen a difference in the expression between the baseline and the end of study biopsy samples from the same individual.

Glutathione peroxidase activity is not an adequate marker for measuring different levels of selenium. None of the samples collected after selenium supplementation showed a significant change in activity as a result from increased selenium in the blood. Apparently, the normal baseline levels of selenium are sufficient for optimal enzyme activity.

The lack of any reportable selenium dependent biomarker modulation suggests that the optimal dose level for a selenium chemoprevention trial is not able to be set. After hearing a presentation on this study in November, 1999, the external scientific advisor committee, recommended finishing the current trial for the planned study period (Phase I), but not initiating another trial (the Phase II portion) unless a better rationale and a more focused approach was developed.

REFERENCES

1. Berggren, M., Gallegos, A., Gasdaska, J. and Powis, G. 1997. Cellular thioredoxin reductase activity is regulated by selenium. Anticancer Research 17:3377-3380.

APPENDICES

Figure legend and Figures 1-7.

APPENDIX

Figure Legends

Figure 1: Histopathology diagnosis correlated with smoking status. For the individual baseline bronchoscopies, multiple biopsies were taken for 53 individuals. The frequency of the most severe bronchial histopathology is correlated with the smoking status obtained from the enrollment questionnaire.

Figure 2: Histopathology diagnosis correlated with the observed white light bronchoscopy. Multiple biopsies were taken for each individual and each biopsy has both the histopathology diagnosis as well as the interpretation by white light (White light classification is: Class I is normal; Class II is characterized by inflammation and/or metaplasia to mild atypia; Class III is characterized by moderate to severe atypia).

Figure 3: Histopathology diagnosis correlated with the observed fluorescent light bronchoscopy. Multiple biopsies were taken for each individual and each biopsy has both the histopathology diagnosis as well as the interpretation by white light (Fluorescent light classification is: Class I is normal; Class II is characterized by inflammation and/or metaplasia to mild atypia; Class III is characterized by moderate to severe atypia).

Figure 4: A consulting pathologist was recruited to validated the diagnosis of our study pathologist. The results show the percent of dysplasia diagnosed in both the biopsies (Path Findings) and in the overall diagnosis (Path Diagnosis).

Figure 5: A comparison of the atypia described during both the white light and fluorescent LIFE bronchoscopy shows that LIFE calls more severe atypia than white light. This is supported by LIFE showing a stronger correlation to the histopathological diagnosis than white light.

Figure 6: Hemoglobin measurements at baseline and after 3 months of selenomethionine supplementation. Total hemoglobin was measured from lysed red blood cells using a kit purchased from Sigma Diagnostics (Cat. No. 525-A). The procedure is based on the oxidation of hemoglobin to methemoglobin and the subsequent conversion of methemoglobin to cyanmethemoglobin by potassium cyanide which has a maximum adsorption at 540 nm. The color intensity measured at 540 nm is proportional to the total hemoglobin concentration. Note that hemoglobin values measured in this way are higher than those obtained with other clinical chemistries. No end of study blood sample was collected from patient 140.

Figure 7: Glutathione peroxidase activity per gram hemoglobin in blood samples taken at baseline and after 3 months of selenomethionine supplementation. Glutathione peroxidase (GPX) activity assay was done in triplicate for each blood sample collected at baseline and after 3 months of selonomethionine supplementation. A separate assay with purified GPX was done as a positive control and assays without sample or the organic peroxide substrate were done as negative controls. The assay is an indirect measure of GPX activity where oxidized glutathione is produced upon reduction of organic peroxide (*tert*-butyl hydroperoxide) and is recycled to its reduced state by exogenously added glutathione reductase (GR). The reduction of oxidized glutathione by GR requires the oxidation of NADPH. The GPX activity is determined by a decrease in adsorption at 340 nm as NADPH is oxidized to NADP. Using the molar extinction coefficent for NADPH (6220 $M^{-1}cm^{-1}$) and the rate of decrease in absorbance at 340 nm, the GPX activity for each sample can be calculated. No end of study blood sample was collected from patient 140.

Figure 1: Histopathology Frequency in Current and Former Smokers

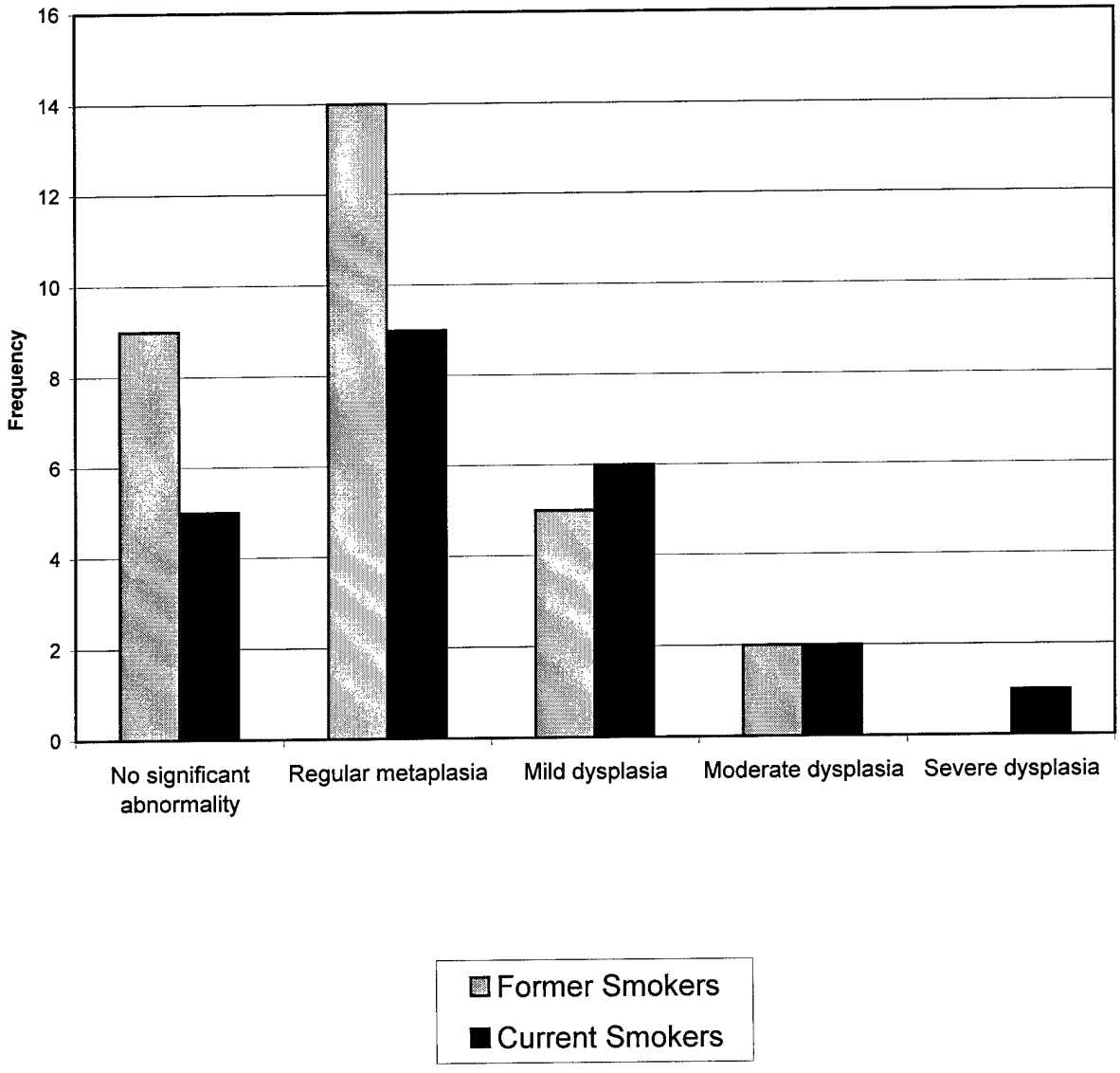

Figure 2: Bronchial Histopathology Findings by White Light Bronchoscopy

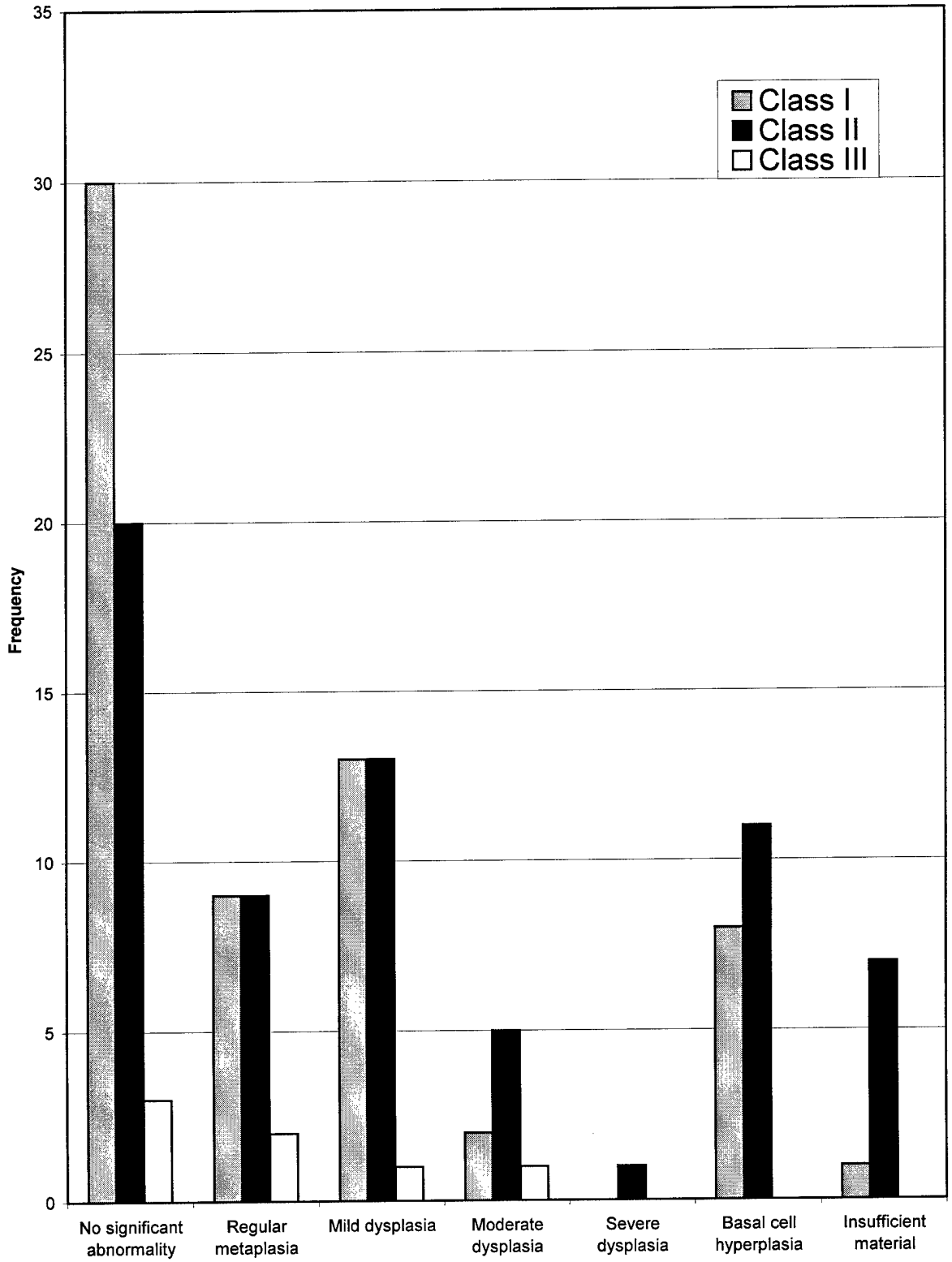

Figure 3: Bronchial Histopathology Findings by Fluorescent Light Bronchoscopy

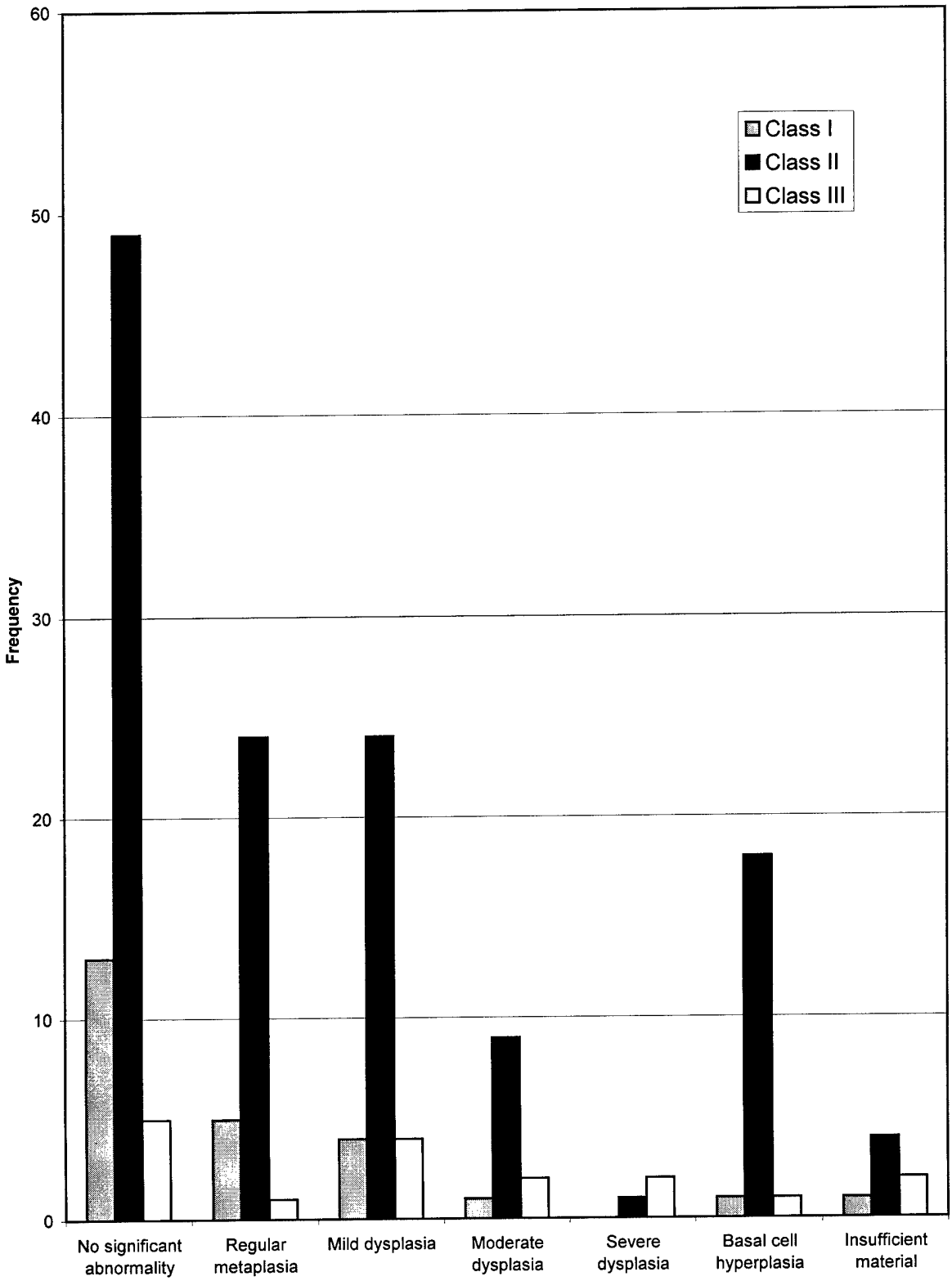

Figure 4: Percent Coded as dysplasia

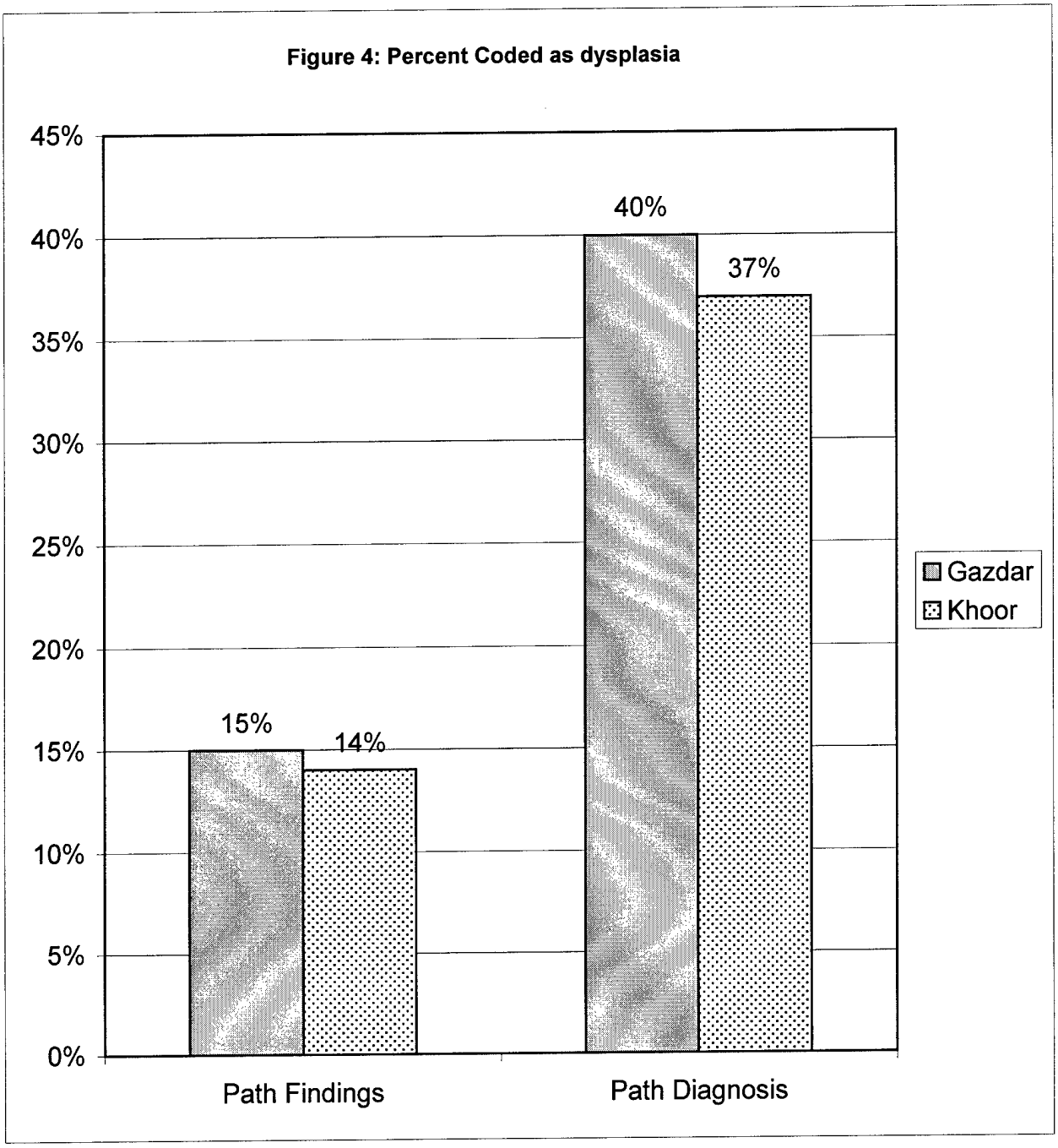

Figure 5: Agreement Between Observations with White Light and LIFE Bronchoscopy

		LIFE CLASS		
		I	II	III
WHITE LIGHT CLASS	I	156	81	5
	II	13	15	2
	III	2	0	2

Observations by LIFE bronchoscopy of 276 endobronchial sites correlated with those by white light bronchoscopy (Spearman correlation coefficient R=0.16, p=0.0087). Agreement was not strong with Kappa statistic=0.108 (95% confidence limits 0.014, 0.20).

Histopathological diagnosis showed a better correlation with LIFE bronchoscopy (R=0.12, p=0.053) than with white light bronchoscopy (R=-0.075, p=0.233).

Figure 6: Hemoglobin Levels

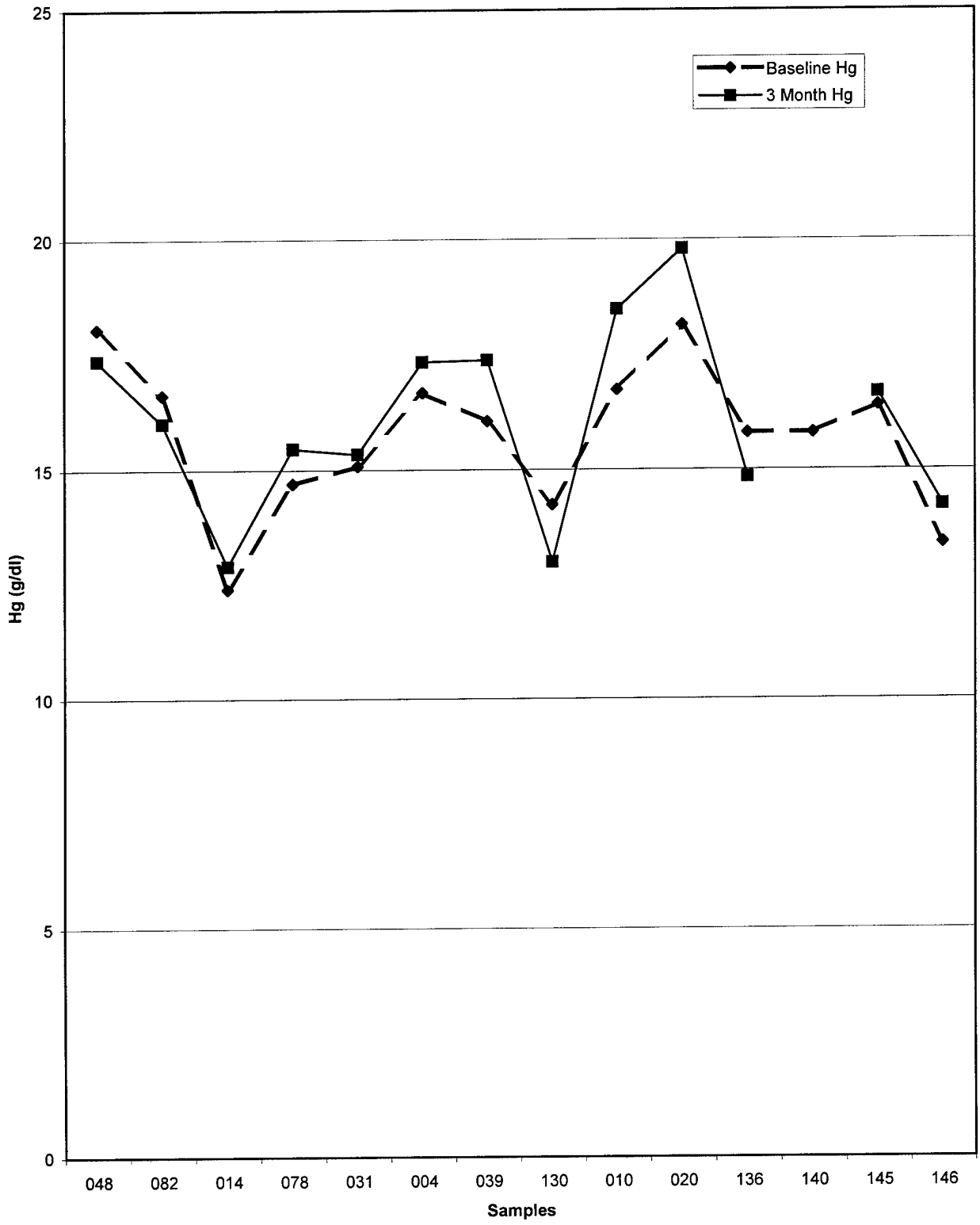

Figure 7: Glutathione Peroxidase Activity